Amendments to the Specification

Please insert the following **new** paragraphs after paragraph [0074]

[0074.1] EXAMPLE 3 – Identification of single nucelotide polymorphic allele information in a highly polymorphic HLA-A genomic DNA molecule using allele specific primers coupled to microspheres

[0074.2] Genomic HLA-A DNA is amplified by PCR as follows. The amplification will be carried out by mixing following components in 20 μl volume in a PCR microtube:

HLA-A locus specific forward and reverse primers 0.25 μM

(In case single-stranded(ss) PCR fragments need to be prepared, the forward primer can have 4 phosphorothioate bonds at their 5' end)

PCR buffers ($MgCL_2$/Amonium Sulfate/Tris)

Deoxnucleotide Triphosphates mix (dNTP), (100uM)

Genomic DNA (100 ng)

Tap DNA Polymerase (1 unit)

[0074.3] PCR is preformed using a PE9600 thermal cycler and following the thermal cycling condition: A mini agarose gel is used to check for successful PCR reaction. Following successful PCR reaction, the PCR primers and dNTPs are degraded according to the teachings of Jingwen Chen et al., 2000 which comprises adding 2 units of Shrimp Alkaline Phosphotase and 4 units of E. Coli exonuclease I to the 20 μl of PCR products and incubating at 37°C for 30 min. The PCR enzymes are inactivated by incubation for 15 min at 99°C.

[0074.4] Single stranded PCR (ssPCR) products are then prepared as taught by Reynolds, et al., 1997. 12 units of T7 gene 6 exonuclease (Amersham Pharmacia) are added to the 20 μl of PCR products and incubated for 30 minutes at 37°C. The loss of double-stranded (ds) PCR fragments can be determined by agarose gel electrophoresis.

[0074.5] Heterosequence sites in the ssPCR products are then determined by synthesizing a basic set of 20 allele-specific primers to perfectly match all allelic sequences at 10 most common polymorphic sequence sites of the ssPCR products. The 3' end base position of each primer should corresponding to a targeted polymorphic base. The 5' end of primer should be modified with an amino linker. Each primer is conjugated with a different Luminex MAP microspheres (Fulton, et al., 1997). All 20 allele-specific primers are mixed with 4μl (>50 ng) of ssPCR products in lx PCR buffer, heated for 2 min at 90°C and annealed for 15 min at 55°C in 50 μl volume.

[0074.6] The primer extension reaction is then conducted by adding 1 unit of DNA Taq Polymerase, dATP/Cy3-dCTP/dGTP/dTTP to the mixture and incubating at 65°C for 20 min. The fluorescence signals associated with each kind of microspheres are measured using Luminex 100 Flow Cytometer and all heterosequence sites are determined. Heterosequence sites will be identified, if two allele-specific primers targeted at the same heterosequence site are positive.

[0074.7] The allele specific DNA fragment are then separated as follows. The following components are mixed together in a microtube, with separate microtubes used for each allele: Biotinylated allele-specific primer/microsphere conjugate targeted to a heterosequence site (500,000 beads)

Ligation primer (0.2 μM)

Ligation buffer

10 units Taq DNA Ligase

20 μl of ssPCR products as produced above

[0074.8] The components are heated for 2 min at 90°C to remove primers, followed by 30 min incubation at 37°C. Strepavidin coated beads are added to the ligation mixture, and incubated for 15 min at room temperature. The components are then heated 5 min at 75-85°C and the microspheres spun down in a microcentrifuge. The supernatant will contain the nonligated materials. The temperature should be set at the level that the ssPCR fragment hybridized to the ligated primers will not be separated while the ssPCR fragment hybridized to the non-ligated primers will be separated.

[0074.9] If the PCR product does not match the 3' end sequence of the allele-specific primer, the ligation will not occur. The PCR products will be released from the beads-SNP primer due to their lower Tm.

[0074.10] If PCR product does match the 3' end sequence of allele-specific primer, the ligation will occur. The ligated SNP-pairing primer will form a strong duplex with PCR product that will have significant higher Tm to sustain 75°C washing step. The supernatant is then removed and the microspheres washed with 500 µl wash buffer (2.5 M tetramethylammonium chloride/0.15% SDS/3mMEDTA/75mM TrisHCL, pH 8.0) by repeating the steps of centrifuging and removing the supernatant. The particular alleles coupled to the microspheres can then be determined by conventional genotyping methods with sequencing being a preferred method.

[0074.11] EXAMPLE 4 – Identification of single nucelotide polymorphic allele information in a highly polymorphic HLA-A genomic DNA molecule using allele specific primers and labeled ddNTPs

[0074.12] HLA-A genomic DNA is amplified as in Example 3. Heterosequence sites are determined as described in Example 3. The allele specific ssPCR products are separated as follows. The following components are mized together in a PCR tube in 20 µl volume:

Allele-specific primer targeted to a heterosequence site (0.2 µM)
Biotin-ddATP (2 µM)
Digoxigenin-ddCTP (2 µM)
4 units Thermal Sequenase
10 µl of ssPCR fragments
4 µl of 5x Thermal Sequenase buffer

[0074.13] For this example, we assume A/C polymorphism at the selected heterosequence site. The amount of ssPCR products can be increased if necessary. Single-base extension reaction is performed by cycling 30-50 times at 94°C for 30 sec, 55° for 30 sec, and 72° for 30 sec. The reaction mix is transferred to a QIAquick Spin Column to remove free Biotin-ddATP and the primers/PCR fragments are eluted in 30 µl Tris buffer in a microcentrifuge tube. 5 µl of

avidin-conjugated magnetic-beads (Dynabeads M-280 Streptavidin) is added and incubated 15 min at room temperature with occasional mixing. A magnet is applied and the supernatant transferred to a second microcentrifuge tube. Digoxigenin-antibody conjugated magnetic beads are added to the second microcentrifuge tube and incubated 15 min at room temperature with occasional mixing. A magnet is applied and the supernatant is removed. The particular alleles coupled to the magnets can then be determined.

[0074.14] EXAMPLE 5 – Haplotyping of separated alleles of Examples 3 and 4

[0074.15] Extension primers for all SNPs are synthesized. The extension primers are designed to be complementary to the single-stranded PCR fragment ending one base short of the polymorphic site (at the 3' end). The 5' end of each primer should be modified with amino linker. For HLA-A haplotyping, a complete set of allele-specific primers could be around 90 different primers. Conjugate each primer with a different Luminex MAP microspheres (Fulton, et al., 1997). 100 different Luminex microspheres could be used to conjugate 100 different primers. The individual microspheres can be identified by its fluorescence color ratio between red and infrared and the fluorescence level. Mix all primer/microsphere conjugates (5,000 microspheres each) in the same microcentrifuge tube containing magnetic beads bound with allele-specific PCR fragments from Examples 3 and 4. Divide equally into 4 PCR tubes. Add 2 μM of biotin-ddATP to the first tube, biotin-ddCTP to the second tube, biotin-ddGTP to the third tube and biotin-ddTTP to the fourth tube. Add the rest of the three kinds of ddNTP to each tube. Add 4 units of Thermal Sequenase and 4 μl of 5xThermal Sequenase buffer. Bring up the final volume to 20 μl. Perform single-base extension reaction by cycling 30-50 times at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec. Add 10 μl of diluted avidin-phycoerythrin solution to each tube. Incubate 10 minutes at room temperature. Measure the green fluorescence signals associated with each primer/microsphere conjugates using Luminex 100 Flow Cytometer. The identity of each beads primer conjugate is determined by the red/infrared fluorescence and the polymorphic nucleotide specificity is determined by the green-fluorescence of phycoerythrin. The recorded results are haplotype/allele-specific which can be analyzed with a specific genotyping software.

[0074.16] EXAMPLE 6 - Identification of single nucelotide polymorphic allele information in a highly polymorphic HLA-A genomic DNA molecule using allele specific primers covalently attached to variable weight molecules

[0074.17] HLA-A genomic DNA is amplified as in Example 3. The allele specific ssPCR products are genotyped as follows. Size tagged SNP extension primers are designed as follows. For each SNP, design a SNP extension primer. The 5' part is consisted of a poly-A tail with a different length of A-bases. The 3' part is 18-22 mer oligonucleotide perfect matching the genomic DNA sequence immediately before the polymorphic base site. The length of A-tail between each SNP primer should differ by 2-4 bases, which allows a clear size distinction by gel-electrophoresis. The first base at the 5' end should be a dye-labeled nucleotide. Each primer by itself could run with slightly different mobility due to different mobility due to different nucleotide composition. This has to be considered when deciding the length of oligo-A-tag added to each primer. 20 individual SNPs with each named as SNP1, SNP2, ...SNP20. 20 SNP extension primers with each named as Pex1, Pex2, ...Pex20. Assume that each SNP extension primer has a similar mobility in a gel electrophoresis. The Pex1 will have 2 adenosine added to it's 5' end and Pex2 will have four adenosine added to it's 5' end. The last SNP extension primer Pex20 will have 40 adenosine attached to it's 5' end. Mix 20 SNP extension primers and verify their clear separation in a ABI 377 sequencer machine. Mix following components together in a microtube in a final volume of 20 μl:

20 SNP extension primers with an unique size-tag (Pex1 to Pex20)
10 μl of ssPCR products
ddNTPs with each dideoxinucleotide labeled with different dyes
4 units of Thermal Sequenase
4 μl of 5xThermal Sequenase buffer

[0074.18] Perform single-base extension reaction by cycling 30-50 times at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec. Add 20 μl of 2x gel loading mix. Load 2 μl on ABI 377. Separate 20 SNP extension primers with single extended base by 8% denaturing PAGE. The nucleotide-specificity at each SNP site is determined by fluorescence signal associated with each SNP extension primer band.